

RADIOAUTOGRAPHIC LOCALIZATION OF SODIUM PUMP SITES IN RABBIT INTESTINE

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ABSTRACT

Direct demonstration of the cellular location of sodium pumping constitutes a key problem in the solution of intestinal sodium absorption. Utilizing silicone-impregnated epoxy sections of freeze-dried, osmium-fixed tissue, ouabain- ^3H and inulin- ^3H light microscope radioautographs have been produced which show that: lateral but not brush border membranes of rabbit small intestine bind ouabain- ^3H (high specific activity) with an affinity so great that a subsequent washing in ouabain-free medium has little effect on binding; lateral membrane binding is not apparent with low specific activity ouabain- ^3H , and inulin- ^3H and ouabain- ^3H (low specific activity) in the cores of the villi do not equilibrate with the intercellular spaces. Preliminary tracer measurements of ouabain- ^3H and inulin- ^{14}C spaces also agree with these findings. As ouabain is a specific inhibitor of active sodium transport, these observations provide direct support for the view that lateral membrane pumping of sodium into the intercellular spaces causes, through osmotic forces on water, a flow of fluid out of these spaces into the interstitium.

INTRODUCTION

The ability of the small intestine to absorb sodium actively is a well-documented phenomenon (26). According to current views (Fig. 1), sodium enters the epithelial cell down its electrochemical gradient primarily by an unidentified mechanism and to a lesser extent by coupling to certain organic solutes (27). Sodium then diffuses across the cell to the lateral membranes, where it is actively pumped by a ouabain-sensitive mechanism (29) into intercellular spaces, thus creating a local hyperosmotic environment between cells. As a result of osmotic forces, water closely follows the sodium fluxes and upon entry into the intercellular spaces creates a hydrostatic pressure, which in turn causes a flow of fluid down the space into the interstitium. Although a basal location for the sodium pump is amply supported by experiment (6, 12, 28, 29, 38), the view that the pump sites are located in the

lateral membranes is still a hypothesis (7, 8) that remains to be substantiated.

The present study undertakes to demonstrate directly the subcellular location of the sodium pump. The success achieved with high resolution radioautography of phlorizin- ^3H in demonstrating the brush border location of intestinal sugar transport sites (30) suggested that a similar approach using high specific activity ouabain- ^3H might reveal the cellular locations of intestinal sodium pump sites.

This study presents high resolution ouabain- ^3H and inulin- ^3H radioautographs of *in vitro* rabbit small intestine which directly support the hypothesis that the lateral membranes of this epithelium possess a limited number of sodium pump sites. Some of these data have been published in a preliminary report (32).

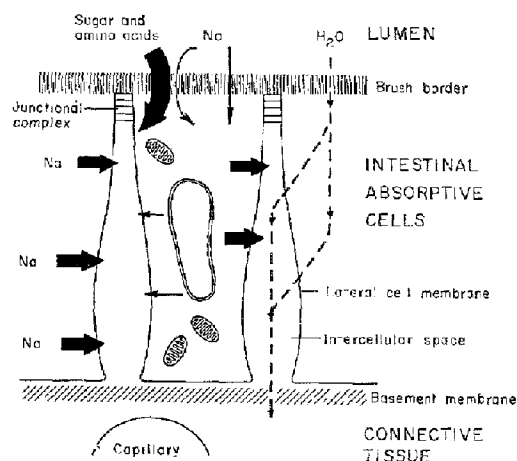


FIGURE 1 Model depicting current concepts of sodium absorption in the intestine. The heavy and thin arrows indicate uphill and downhill movement. Sodium moves downhill across the brush border coupled to sugar and amino acid transport (curved arrows) and via a non-coupled path (straight arrow). Sodium, sugar, and amino acids then diffuse transeellularly to the lateral membranes where sodium is actively pumped into the intercellular spaces and where sugars and amino acids enter via facilitated diffusion (thin straight arrows). Osmotic gradients generated by sodium movement cause water (dashed arrows) to move passively across the epithelium.

MATERIALS AND METHODS

Tissue Preparation

Intestine was obtained from New Zealand rabbit (2.5–3.2 kg) maintained on a standard laboratory diet. Immediately after a lethal injection of sodium pentobarbital, the abdomen was opened and the intestine removed and washed with iced saline. After trimming away the remaining mesentery, everted sacs (37), rings (5), and mucosal sheets (5) were prepared as required for the particular experiment. The tissue was preincubated for 10 min at 37°C in a balanced salt medium (17), buffered with bicarbonate (5% CO₂, 95% O₂) to pH 7.4, and then transferred to medium containing 25 μCi/ml of specified radioactive test compounds. In some experiments the tissue was subsequently incubated in medium free of test compounds.

Test Compounds

Tritiated ouabain (11.7 Ci/mole, lot 184-239), inulin-³H (137 μCi/mg, lot 96-284-25), and inulin-¹⁴C (1.75 Ci/mg, lot 126-45-7C) were purchased from the New England Nuclear Corp., Boston, Mass. The desired medium concentrations of ouabain-³H were

obtained by adding unlabeled ouabain purchased from Mann Research Labs., Inc., New York.

Radioautography

The following is a brief summary of the radioautographic procedures previously reported in detail (31): at the completion of incubation the tissue was cut into several small bits and quickly frozen in liquid propane cooled to -175°C with liquid nitrogen. The frozen bits of tissue were then freeze dried at low temperatures, fixed in osmium tetroxide vapor, and vacuum embedded in a mixture of silicone oil and epoxy-resin. Sections (1–2 μ thick) cut with glass knives and collected over water were then placed on microscope slides and coated with liquid photographic emulsion (Kodak NTB-2) and exposed 4–25 days. After development, the sections were stained with basic fuchsin and examined by bright field microscopy.

Resolution tests of this technique (31, 33) have shown that 97 ± 3% of the silver grains produced by a radioactive 2 μ band fall over that band. In the present study, this resolution is essential, for the width of the epithelial cells at their base (≈ 4 μ) is only slightly larger than the intercellular spaces in this region. Lesser resolution could easily lead to confusion of lateral membrane binding with cellular entry.

As the results show, diffusion of either ouabain or inulin during processing for radioautography was negligible. Indeed, from the physical similarity of these compounds to galactose, mannitol, and phlorizin, which have been shown to behave reliably with this technique (30, 31, 33), the presence of diffusion artifacts would have been quite unexpected.

In some instances, a quantitative comparison of ouabain content among medium, cytoplasm, and brush border proved of value (Table 1). These data were obtained by computing grain densities (grains/μ²) of a given tissue structure from micrographs similar to those shown in the Results. Individual ratios used to compute the averages given in Table 1 are based on 100 or more grains for each structure.

Preliminary Measurements of Ouabain-³H and Inulin-¹⁴C Spaces

Everted sacs and mucosal sheets were incubated in media containing both ouabain-³H and inulin-¹⁴C. At the end of incubation the tissue was homogenized, precipitated with 10% trichloroacetic acid, and warmed to 100°C. After cooling, a portion was recovered, neutralized to pH 6.5, and analyzed by scintillation spectrometry. The scintillation mixture contained 4 ml of sample plus 11 ml of Aquasol (New England Nuclear Corp.). Tissue measurements of ouabain-³H and inulin-¹⁴C were then compared with those from a similarly treated portion of incubation

medium (Table II). Estimates of ouabain-³H and inulin-¹⁴C in control tissues differed less than 5 and 3% from the predetermined amounts added.

RESULTS

In order to evaluate the possibility of mucosal entry and brush border binding, radioautographs were prepared from everted sacs incubated 30 min in 5×10^{-4} and 10^{-6} M ouabain-³H. The radioautograph of Fig. 2 is typical of these results. Very few grains are ever found over the tissue, even with long radioautographic exposures which produce exceptionally high grain densities over the medium. Clearly, ouabain does not easily permeate brush border membranes. A more quantitative assessment of permeation based on grain density measurements (Table I) indicates that at both high and low concentrations of ouabain the cellular concentration is less than 2% of the medium. This figure agrees rather well with estimates of *in vivo* intestinal permeability to ouabain in the guinea pig (19). Further confirmation of brush border impermeability to ouabain was obtained from tracer analyses of everted sacs incubated in inulin-¹⁴C and ouabain-³H. The preliminary data of Table II show that after 30 min the ouabain distribution space¹ of everted sacs differed insignificantly from that of the extracellular marker inulin.

¹ Defined as the amount per gram of tissue divided by the amount per milliliter of incubation medium. If a

Since the total ouabain content of the sac can be accounted for by medium adhering to its surface, brush border binding as well as cellular entry must be negligible. The absence of brush border binding is directly observable in ouabain-³H radioautographs (Figs. 2-7). In phlorizin-³H radioautographs of hamster (30) and human (33), intestine brush border binding was distinguished as an increased density of grains over the brush border band of the epithelium, in none of the ouabain-³H radioautographs examined was there any suggestion of a similar brush border accumulation.

Quantitative comparison of grain density over brush border and medium alone (Table I) indicates that the ouabain content of the brush border is about 18% of that in the medium. Since the extracellular fraction of the brush border band (31) differs insignificantly from this figure, these radioautograph observations are entirely consistent with the conclusion that brush border membranes do not bind ouabain.

The binding of ouabain to basal membranes of the epithelium was first examined in intestinal rings. As the radioautograph of Fig. 3 shows, ouabain penetrates the intercellular spaces of the epithelium in addition to the villar cores when the serosal side of the intestine is exposed to incubation

compound is membrane bound, its distribution space is a derived quantity since this space corresponds to no real tissue volume.

FIGURE 2 Bright field view of radioautograph showing several villi (*V*) of an everted sac (animal OBG-1) incubated 30 min in 10^{-6} M ouabain-³H. The brush border (*B*) of the epithelium (*E*) is bathed by incubation medium (*M*). At its basal limit the epithelium is bounded by the connective tissue core (*VC*) of the villus. The freezing and drying procedures often produce fractures (*F*) in the media, particularly along the medium-brush border interface. The silver grains of the overlying photographic emulsion, which reflect the distribution of ouabain, are barely discernible as discrete black dots at this magnification. The very high grain density resulting from a prolonged exposure has given the medium a stippled appearance. The very few grains over the villi show that little ouabain has penetrated the brush border. The abrupt change in grain density between medium and areas which contain only epoxy resin (*R*) is indicative of the radioautographic resolution. Scale: $10 \mu. \times 550$.

FIGURE 3 Radioautograph of intestinal ring (animal OBG-1) incubated 30 min in 10^{-6} M ouabain-³H. Because of the increase in magnification as compared to Fig. 2, the individual silver grains and intercellular spaces (*I*) are easily distinguished. The appearance of silver grains over the connective tissue core (*VC*) of the villus is to be expected because the serosal side of the intestine is exposed to incubation medium. The much lower ouabain content of the villar core as compared with that of the medium reflects the considerable resistance of the muscle layers to ouabain entry. Note that most of the grains over epithelium are located along the borders of the intercellular spaces (*I*). This is especially evident where the filaments of basal cytoplasm span these spaces. Moreover, the grain density in the intercellular space region appears to be considerably higher than that of the villar cores. Although the brush border band (*B*) possesses considerable radioactivity, it is still less than that of the medium (*M*). Scale: $5 \mu. \times 1800$.

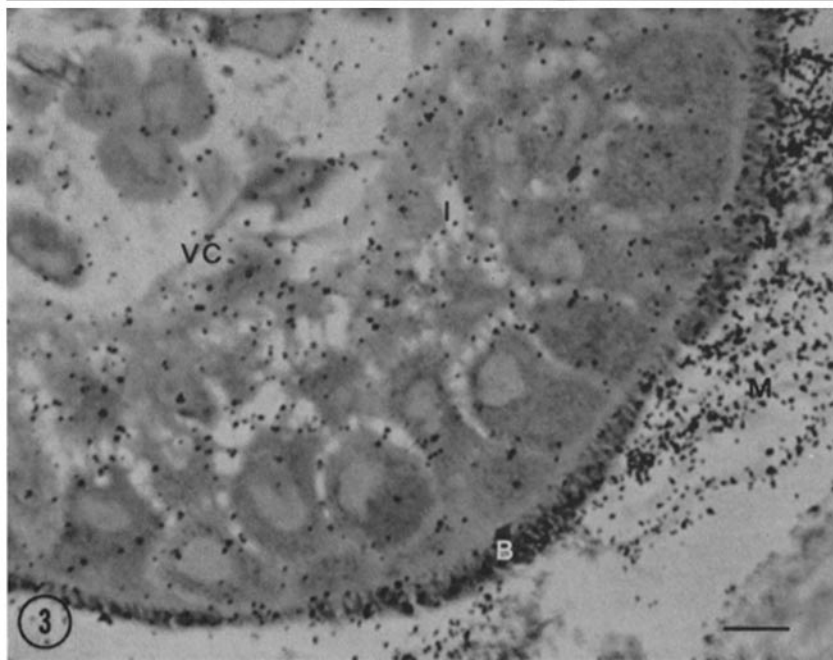
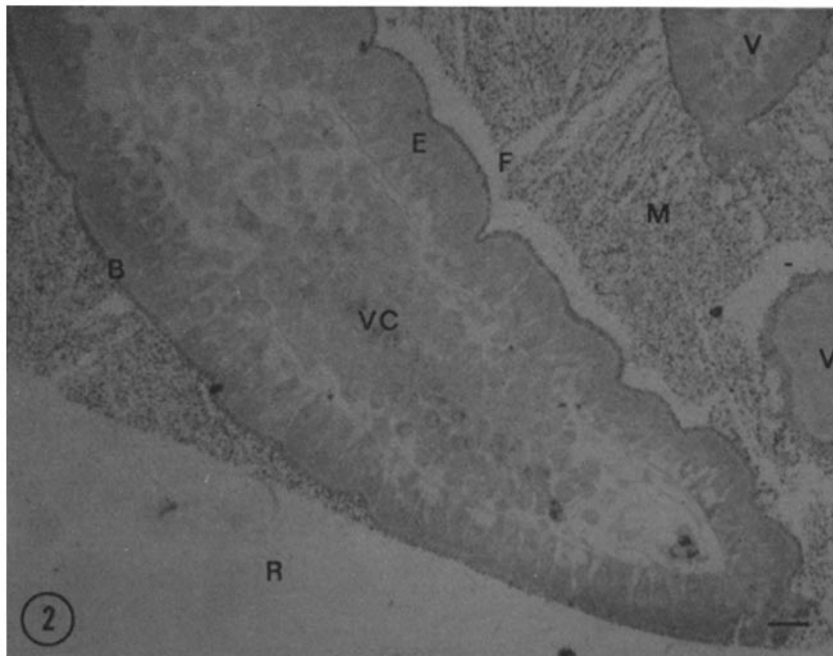


TABLE I
Grain Density Measurements for Ouabain-³H Radioautographs

Animal	Ouabain (medium)	Relative grain densities*		
		Medium ‡	Cell	Brush border
OBG-1 §	10 ⁻⁶ M	1.0 ± 0.23	0.014 ± 0.004	0.17 ± 0.02
OBG-1	5 × 10 ⁻⁴ M	1.0 ± 0.20	0.011 ± 0.003	0.20 ± 0.03
OBG-2 (Fig. 4)	10 ⁻⁶ M	1.0 ± 0.25		0.17 ± 0.04

* Reported values are averages of 10 individual measurements ± SD, an individual measurement is based on 100 or more grains per structure.

‡ The medium grain density was arbitrarily set at 1.0; the standard deviations of these measurements are a measure of the precision of the technique.

§ Same animal as in Fig. 2; however, measurements were made on radioautographs of lower grain densities. Radioautographs at higher medium concentrations are not shown.

TABLE II
Tracer Analysis of Ouabain-³H and Inulin-¹⁴C Distribution Spaces*
in Everted Sacs and Mucosal Sheets

Animal	Ouabain (medium)	Sacs † distribution spaces §		Sheets ‡ distribution spaces §	
		Inulin- ¹⁴ C	Ouabain- ³ H	Inulin- ¹⁴ C	Ouabain- ³ H
		<i>ml/g</i>		<i>ml/g</i>	
OAG-1	10 ⁻⁶ M	0.042	0.037	0.149	0.233
OAG-1	5 × 10 ⁻⁴ M	0.042	0.047	0.198	0.186
OAG-2	10 ⁻⁶ M	0.048	0.044	0.220	0.965
OAG-2	5 × 10 ⁻⁴ M	0.045	0.039	0.205	0.217

* Calculated as specific radiolabel (¹⁴C, ³H) per gram of blotted tissue divided by specific radiolabel per milliliter of incubation medium.

‡ Incubated for 30 min in 100 mg% inulin-¹⁴C and above concentrations of ouabain-³H.

§ Averages of three individual measurements from adjacent segments of distal ileum.

medium. Equilibration, however, between the extracellular space of the villi and the incubation medium, even after 30 min, is still far from complete. Because of this poor penetration of the serosal compartment, further experiments were conducted in mucosal sheets in which the heavy muscle layers are absent. With this preparation, 30-min incubations gave almost complete equilibration between villar core and medium (Fig. 4).

The most striking feature of radioautographs prepared from either rings or mucosal sheets incubated in 10⁻⁶ M ouabain (Figs. 3, 4, and 5) is the large accumulation of grains either along or in close proximity to the boundaries of the intercellular spaces. This is especially evident in Fig. 3, where many of the grains appear in clusters over the cytoplasmic filaments penetrating the intercellular spaces. Occasionally, a few grains are found over the central areas of the cells; whether

these grains truly represent intracellular ouabain is not clear. Because the sections are from 1 to 2 μ thick and the cells 5 to 7 μ in cross-section, the possibility that these cellular areas include a small amount of lateral membrane cannot be excluded. In any case, the rareness of grains over the central cellular areas indicates that the lateral membranes of the epithelium are just as impermeable to ouabain as the brush border membranes.

Binding of ouabain to the lateral membranes of the intercellular channels was further examined by subjecting mucosal sheets which had been incubated in 10⁻⁶ M ouabain to a subsequent 30 min incubation in ouabain-free medium. This wash treatment had little effect on bound ouabain, as Figs. 4 b and 5 show, the accumulation of grains over the intercellular spaces is just as great as that in the unwashed tissue. The primary effect of the ouabain-free medium is to enhance the radioauto-

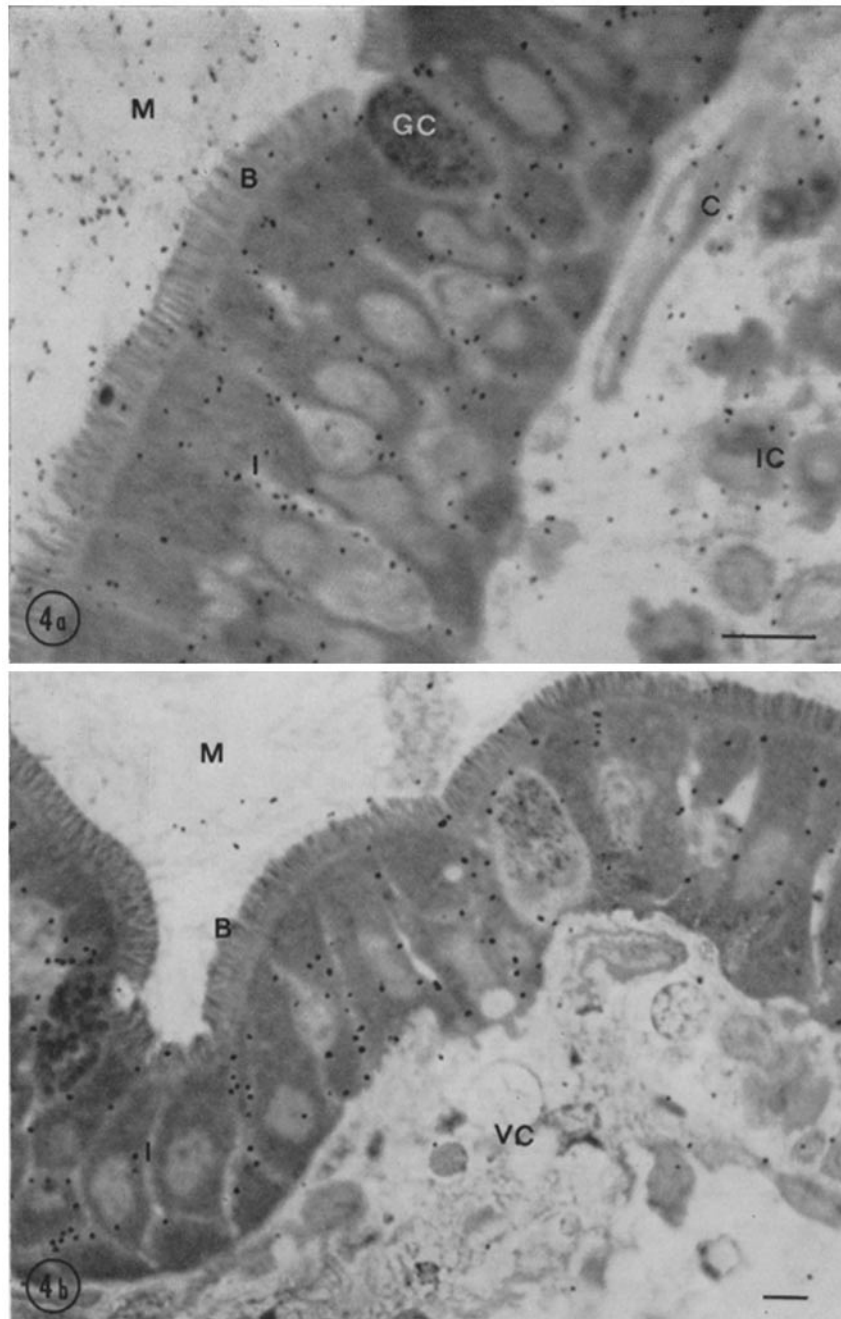


FIGURE 4 (a) Ouabain- ^3H radioautograph of a mucosal sheet taken from another animal (OBG-2), incubation was for 30 min at 10^{-6} M. With removal of the muscle coat, the ouabain concentration of the villar core is nearly equal to that of the medium. The grain density over the medium as compared with that of Figs. 2 and 3 is due to a shorter radioautographic exposure. As in the previous experiment (Fig. 3), there is a distinct accumulation of ouabain over the intercellular spaces (I). The grain density of the brush border (B) is clearly less than that of the medium (M). The occurrence of silver grains over the epithelium which is not in close proximity to intercellular spaces is quite rare. Similarly, goblet (GC) and interstitial cells (IC) are relatively free of ouabain; capillaries (C), however, appear to be quite permeable to ouabain. Scale: 5μ . $\times 2500$. (b) Same animal and experimental conditions as in Fig. 4a except that the incubation in ouabain- ^3H was followed by a 30 min incubation in ouabain-free medium. The medium (M), brush border (B), and villar core (VC) are nearly ouabain-free. The intercellular spaces (I), however, have lost little if any of their original activity, clearly indicating a tight binding of glycoside in this region. Note again the very few silver grains over the epithelial cells. Scale: 5μ . $\times 1200$.

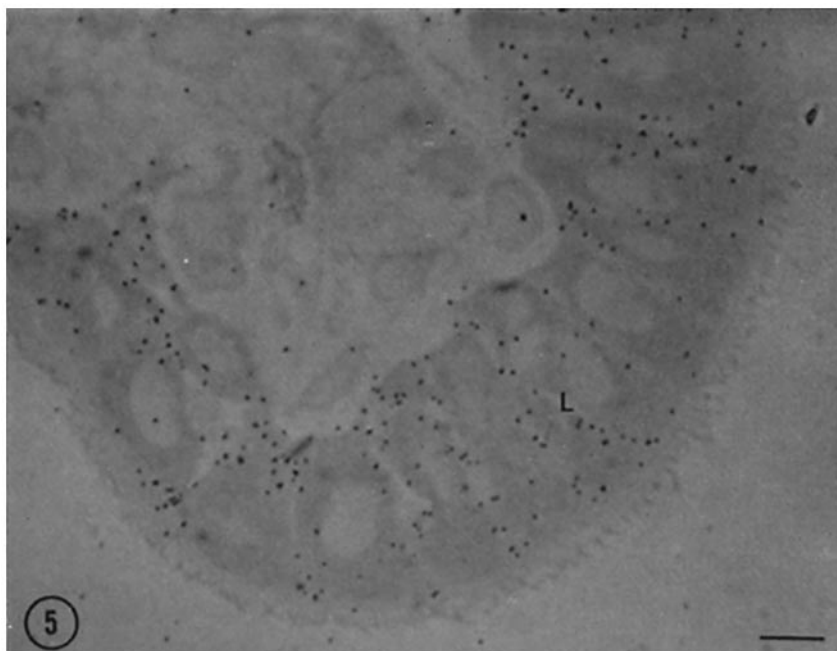


FIGURE 5 Radioautograph of a mucosal sheet from a third animal (OBG-3); experimental conditions were the same as in Fig. 4 *b*: 30 min incubation in 10^{-6} M ouabain- 3 H followed by 30 min incubation in ouabain-free media. As in the previous experiment (Fig. 4 *b*), nearly all of the ouabain has been washed out of the medium and villar core, leaving only that bound to the lateral membranes of the intercellular spaces. In some cases the grains form a line (*L*) tracing the course of the intercellular channel to its origin at the junctional complex. Scale: 5μ . $\times 1800$.

graphic contrast (Figs 4 *b* and 5) by washing out most of the activity in the incubation medium and extracellular space of the villi. The virtual absence of grains over the brush border is even stronger evidence that brush border membranes do not bind ouabain.

If indeed the accumulation of grains in the proximity of the intercellular spaces arises from ouabain binding to a limited number of lateral membrane sites, then raising the concentration by addition of unlabeled ouabain should, through competition, greatly attenuate this accumulation. To test this hypothesis control mucosal strips were incubated 30 min in 10^{-6} M ouabain- 3 H and washed 30 min in ouabain-free medium (Fig. 5). An adjacent strip of mucosa was treated identically except that the concentration of the medium was raised to 5×10^{-4} M with unlabeled ouabain (Fig. 6). Radioautographs were prepared from both washed and unwashed tissue. The control tissue exhibited the usual heavy accumulation of grains along the intercellular spaces; however, there was little if

any accumulation in either the unwashed (Fig. 6) or washed tissue (not shown) incubated at the higher concentration.

The grain density pattern in the high ouabain tissue is remarkably similar to that of tissue incubated 30 min in inulin- 3 H (Fig. 7). The rareness of grains over the intercellular spaces in both tissues (Figs. 6 and 7) indicates that compounds which enter the villar core via the serosal pathway do not readily equilibrate with the fluid of the intercellular spaces. The lack of equilibration may very well be caused by a flow of fluid out of these channels, as proposed in the Introduction (Fig. 1). Most importantly, these experiments (Figs 6 and 7) show that the accumulation of grains along the channels at low ouabain concentrations cannot be accounted for by equilibration between intercellular and villar extracellular fluids.

Finally, one would predict from these radioautographic observations that mucosal strips incubated in low concentrations of ouabain- 3 H (high specific activity) and inulin- 14 C would have, as a result of

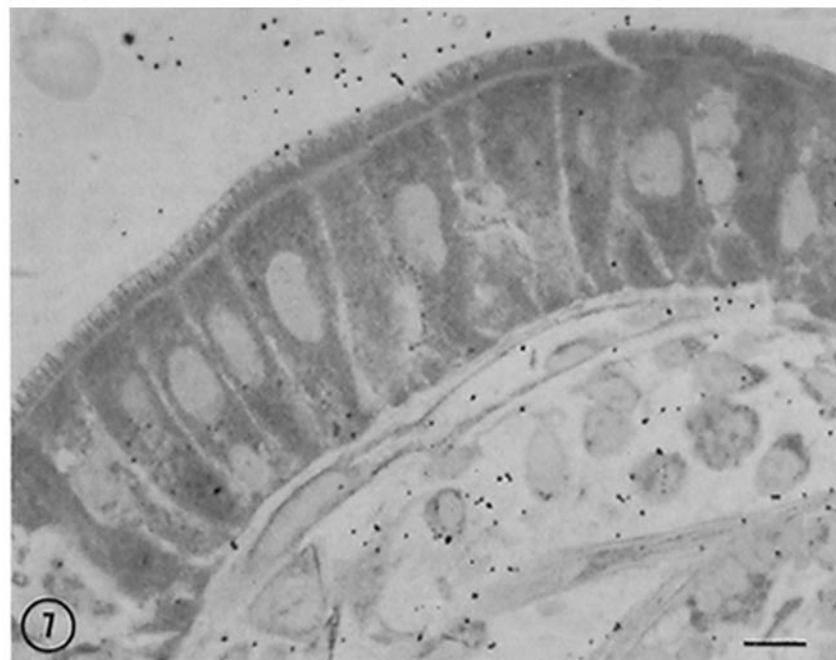
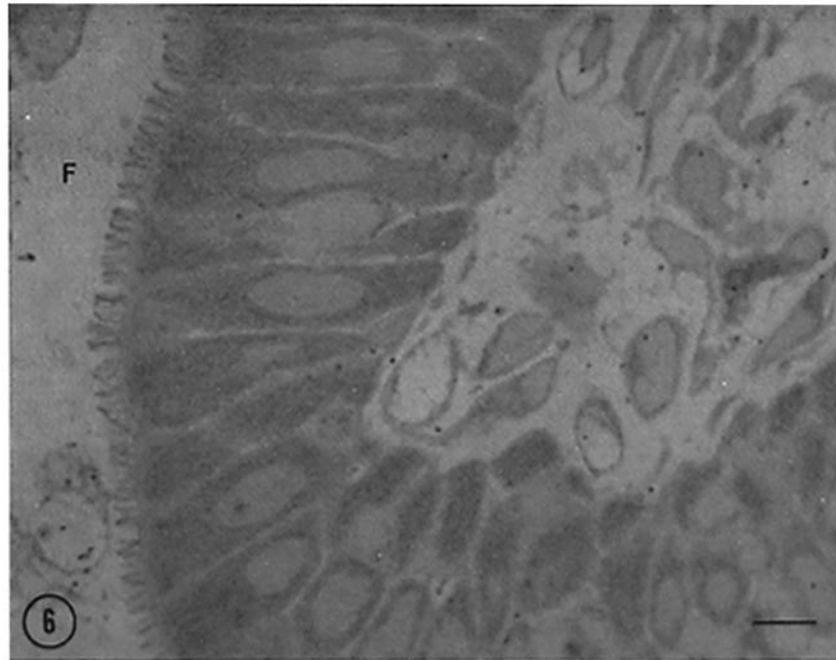


FIGURE 6 Mucosal sheet from same animal (OBG-3) in Fig. 5 incubated 30 min in ouabain- ^3H whose concentration was raised to $5 \times 10^{-4} \mu\text{M}$ with unlabeled ouabain. Although there is ample entry of ouabain into the villar core, there is little indication of accumulation over the intercellular spaces. The light region (F) adjacent to the brush border is a freezing fracture. Scale: $5 \mu. \times 1700$.

FIGURE 7 Mucosal sheet from a fourth animal, which was incubated 30 min in inulin- ^3H (1 mg/ml). The inulin activities of the medium and villar core are nearly identical. As expected, there are very few grains over the epithelial cells. Most noteworthy, however, is the absence of grains over the intercellular spaces. Scale: $5 \mu. \times 1700$.

lateral membrane binding, a ouabain distribution space significantly larger than that of inulin, and that raising the concentration with unlabeled ouabain would reduce this space to that of inulin. As the tracer analysis data of Table II show, precisely this result was obtained. A 30 min exposure to 10^{-6} M ouabain- ^3H and inulin- ^{14}C gave a distribution space 2–4 times that of inulin, while a similar exposure at 5×10^{-4} M (low specific activity) gave a distribution space equivalent to that of inulin. Therefore, these different methods of evaluating binding give consistent results.

DISCUSSION

It is apparent from the results of this study that lateral membranes of the small intestinal epithelium possess a limited number of sites that bind ouabain with a high affinity. The question of primary importance is whether or not the binding is mostly to sodium pump sites rather than nonspecific sites of the membrane. Several lines of evidence support the argument for specific binding.

Certainly, the high affinity and specificity of ouabain for the sodium pump (13, 29) is consistent with binding specifically to sodium pump sites. Reagents which bind indiscriminately to common chemical groups of the membrane are characteristically nonspecific inhibitors of membrane permeability (25). Recent studies of ouabain binding in the erythrocyte (10, 14, 15) have indicated that as much as 50% of the total binding may be to sites other than the sodium pump. Values this large, however, are probably peculiar to the erythrocyte because of the small number of pump sites (100 or less per cell). In tissues known to have considerably higher sodium pumping rates than that of the erythrocyte, estimates of sodium pump site densities (1, 21) based on ouabain binding are 100 or more times higher than estimates for the erythrocyte (10, 11, 14, 15). As the rate of sodium transport in the small intestine is also large, it is reasonable to assume that the fraction of nonspecific binding is negligible.

Even more compelling evidence for specific binding is the differential pattern of ouabain binding observed in this study. If ouabain binds predominantly to sodium pump sites, then one should predict binding to lateral cell membranes but not to brush border membranes, because this glycoside is inhibitory only when it has access to the serosal side of the intestine (6, 29). A similar conclusion may be reached from estimates of intestinal

sodium electrochemical potentials (12, 24, 26, 28, 29, 38), for these indicate that the basal but not the brush border cell membrane requires a sodium pump to effect net absorption. Perhaps it should be emphasized that while these arguments support the hypothesis of specific binding, they do not constitute its proof. Brush border and basal cell membranes differ in other properties (e.g., enzymatic activity), and therefore they could conceivably possess a similar differential distribution of nonspecific binding sites.

Active pumping of sodium across lateral membranes into intercellular spaces (Fig. 1) is a key requirement of the "double membrane" model (7) of salt and water transport in epithelia and its corollary the "standing gradient" hypothesis (8). Except for the report of a hyperosmotic absorbate in the intercellular channels of *Periplaneta* rectal pads (36), and the several observations of intercellular precipitates of sodium pyroantimonate in various other epithelia (3, 16), most of the evidence supporting these hypotheses has been indirect (18, 31, 35). The evidence from this study that ouabain binds predominately to sodium pump sites of the lateral membranes of the small intestinal epithelium constitutes a direct critical support for these hypotheses.

Analysis (9) of a right cylinder analog of the intercellular channels suggests that for the absorbate to emerge isosmotic to the luminal fluid as generally occurs in the intestine (4, 20), the membrane density of sodium pump sites should be greatest in the most apical, or junctional, complex region of the channel. No such asymmetry was obvious in the ouabain radioautographs. However, the number of grains along a single channel is so small that the large variation arising from the random nature of nuclear decay would most likely obscure variations in density along the space less than a factor of two between apical and basal regions. In addition, estimates of binding per unit length of intercellular space in light microscope radioautographs are subject to further uncertainties because of the poorly defined limits of these spaces.

In view of the recent reports of (Na^+-K^+) -stimulated ATPase activity in the isolated brush border fraction of the small intestine (2, 23, 34), the differential pattern of ouabain binding merits additional comment. If brush border membranes do possess a (Na^+-K^+) -stimulated ATPase, the absence of ouabain binding would be unexpected,

for it is generally believed that the ouabain inhibitory sites of this ATPase and the sodium pump are identical (evidence for this hypothesis has been recently reviewed by Glynn, 13). The brush border membranes may indeed possess ouabain-binding sites, but either so few or of such low affinity that binding could not be detected by the methods of this study. Although highly speculative, a possible explanation for the disparity is that the ouabain-binding sites of the purported brush border (Na^+ - K^+)-stimulated ATPase are oriented toward the intracellular face of the membrane in contrast to their usually extracellular orientation. A more probable explanation (23) is that the (Na^+ - K^+)-stimulated activity of the brush border preparation is due to contamination. Lateral membrane tags attached to the junctional complex are quite evident in electron micrographs of the isolated brush border (22, 23). Whether or not this contamination can account for all of the observed activity is unknown.

In conclusion, it should be noted that the present radioautographic method has provided the unique opportunity of directly viewing the cellular location of sodium pump sites. The demonstration that these sites appear only along the lateral membranes clearly emphasizes the dependence of tissue function on cellular structure and membrane specialization.

Radioautography has not yielded quantitative estimates of lateral membrane binding. Therefore it has not been possible to estimate the number of sodium pump sites per cell and thence their turnover rates. Quantitative problems of this nature are best solved by methods of tracer analysis and must be forthcoming if a more complete understanding of intestinal sodium absorption is to be achieved.

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